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Diversity of Shiga Toxin-Producing *Escherichia coli* (STEC) O26:H11 Strains Examined via *stx* Subtypes and Insertion Sites of Stx and EspK Bacteriophages

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Shiga toxin-producing *Escherichia coli* (STEC) is a food-borne pathogen that may be responsible for severe human infections. Only a limited number of serotypes, including O26:H11, are involved in the majority of serious cases and outbreaks. The main virulence factors, Shiga toxins (Stx), are encoded by bacteriophages. Seventy-four STEC O26:H11 strains of various origins (including human, dairy, and cattle) were characterized for their *stx* subtypes and Stx phage chromosomal insertion sites. The majority of food and cattle strains possessed the stx_{1a} subtype, while human strains carried mainly stx_{1a} or stx_{2a}. The *wrbA* and *yehV* genes were the main Stx phage insertion sites in STEC O26:H11, followed distantly by *yecE* and *sbcB*. Interestingly, the occurrence of Stx phages inserted in the *yecE* gene was low in dairy strains. In most of the 29 *stx*-negative *E. coli* O26:H11 strains also studied here, these bacterial insertion sites were vacant. Multilocus sequence typing of 20 *stx*-positive or *stx*-negative *E. coli* O26:H11 strains showed that they were distributed into two phylogenetic groups defined by sequence type 21 (ST21) and ST29. Finally, an EspK-carrying phage was found inserted in the *ssrA* gene in the majority of the STEC O26:H11 strains but in only a minority of the *stx*-negative *E. coli* O26:H11 strains. The differences in the *stx* subtypes and Stx phage insertion sites observed in STEC O26:H11 according to their origin might reflect that strains circulating in cattle and foods are clonally distinct from those isolated from human patients.

Shiga toxin-producing *Escherichia coli* (STEC) strains are a diverse group of food-borne pathogens, including enterohemorrhagic *E. coli* (EHEC), that are responsible for diseases in humans such as diarrhea, hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS) (1). The most important natural reservoirs of STEC are cattle (2). Transmission to humans occurs through food, water, and direct contact with animals or their environment. A large number of STEC serotypes are known. Although O157:H7 is the most important, four non-O157 STEC serotypes, O26:H11, O103:H2, O145:H28, and O111:H8, have emerged as leading causes of infection. Serotype O26:H11 was first identified as a cause of HUS in 1983 (3, 4) and is the second most frequently detected serotype in Europe, accounting for 12% of all clinical EHEC isolates in 2012 (5). It has also been isolated in the United States and several countries in Europe (6–8).

Shiga toxins (Stx) are considered the major virulence factor of STEC (9, 10). There are two Stx groups, Stx1 and Stx2, which are divided into three (a, c, and d) and seven (a to g) subtypes, respectively (11, 12). STEC strains carry Stx1, Stx2, or both. However, Stx2 is more often associated with severe disease (12, 13). In the mid-1990s, a new highly virulent stx_{2a}-positive *E. coli* O26:H11 clone of sequence type 29 (ST29) emerged in Europe (6). The genetic information for the production of Stx1 and Stx2 is located in the genome of lambdoid prophages (2, 14–17). During infection of *E. coli* cells, Stx phages can insert their DNA into specific chromosomal sites and remain silent (16, 18), allowing their bacterial hosts to survive as lysogenic strains. In contrast to many genetic elements that are frequently integrated within tRNA genes (19), Stx phages insert their DNA preferably into genes from the basic genetic equipment of the *E. coli* chromosome (20). Nine Stx phage insertion sites have been described, including *wrbA*, which codes for a tryptophan repressor-binding protein (21); *yehV*, which codes for a transcriptional regulator (22, 23); *yecE*, whose function is unknown (24); *sbcB*, which produces an exonuclease (25, 26); *Z2577*, which codes for an oxidoreductase (27); *ssrA*, which encodes a tmRNA (28, 29); *prfC*, which encodes peptide chain release factor 3; *argW*, which codes for tRNA-Arg; and the *torS-torT* intergenic region (30–32). The *ssrA* gene is also known as an insertion site for EspK phages carrying the type III effector EspK-encoding gene (33). By studying the Stx phage insertion sites among 606 EHEC O157 strains of various geographic origins, Mellor et al. showed that the genotype *wrbA yehV* stx_1 stx_2 was more frequent in the United States while the profile *argW sbcB*

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yehV stx_1 stx_{2c} was more prevalent in Australia, suggesting a divergent evolution of EHEC O157 in Australia and the United States (34). Prophages of non-O157 EHEC strains were also shown to be remarkably divergent in their structure and integration sites from those of EHEC O157 (Sakai strain) (30).

Considered highly mobile genetic elements, Stx phages are involved in the horizontal transfer of *stx* genes (20, 35, 36). Loss of Stx phage and hence of the *stx* genes by EHEC O26:H11 was also shown to occur *in vitro* and *in vivo* in humans, leading to the production of *stx*-negative *E. coli* O26:H11 (37, 38). Contamination of raw-milk cheeses with STEC and *stx*-negative *E. coli* O26:H11 was reported previously (39). It was noteworthy that O26:H11 was the *E. coli* serotype most frequently found in the cheeses studied. The presence of STEC and *stx*-negative *E. coli* O26:H11 strains has also been detected in food products during French surveillance plans (40), with samples containing either *stx*-positive or *stx*-negative *E. coli* strains identified in equivalent proportions. In contrast, the average annual incidence of HUS cases in France remains low (<0.8/100,000 children under 15 years old [41]), with a predominance of the O157:H7 serotype, therefore questioning the virulence level of STEC O26:H11 isolates contaminating these foodstuffs. It is not known whether the *stx*-negative *E. coli* O26:H11 detected in foods originated from STEC O26:H11 upon the loss of Stx phages, i.e., in cattle or other animal hosts, within the food matrix, or during isolation in a laboratory. Consequently, assessment of food safety by molecular screening methods such as ISO/TS 13136 can be problematic when food enrichment broths are found *stx* positive by PCR and STEC isolation attempts only lead to the recovery of *stx*-negative *E. coli* O26:H11 strains. Indeed, when such diagnostic results are obtained, the presence of STEC O26:H11 in food and loss of the Stx phage during enrichment and strain isolation steps cannot be excluded.

In this study, 74 STEC O26:H11 strains were selected and analyzed for their *stx* subtypes and Stx phage insertion sites, and the results obtained were compared according to strain origins, i.e., human, dairy, and cattle. An additional group of 29 *stx*-negative *E. coli* O26:H11 strains was also studied to evaluate the state of Stx phage insertion sites.

MATERIALS AND METHODS

Bacterial strains. Seventy-four STEC O26:H11 isolates from humans ($n = 31$), dairy products ($n = 31$), and cattle ($n = 12$) and 29 *stx*-negative *E. coli* O26:H11 isolates from humans ($n = 8$), dairy products ($n = 9$), and cattle feces or ground beef ($n = 12$) were used in this study (see Tables 2 and 3). Bacterial strains of dairy and cattle origins were isolated in Europe (mainly in France) between 2007 and 2012 and those of human origin were isolated between 1994 and 2011 (except for two strains, H19 and H30, that were isolated in 1977 in Canada). *E. coli* strains were cultivated in tryptone soy broth-yeast extract at 37°C overnight. Bacterial DNA was extracted with the InstaGene Matrix 100 as described by the supplier (Bio-Rad Laboratories, Marnes-la-Coquette, France) and stored either at 4°C before PCR analysis or at −20°C for longer storage.

PCR techniques. Subtyping of *stx* genes allowing the identification of three subtypes of the stx_1 gene (stx_{1a}, stx_{1c}, and stx_{1d}), and seven subtypes of the stx_2 gene (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}) was performed by conventional PCR as described by Scheutz et al. (11), with a 9700AB thermocycler (Applied Biosystems).

Amplification of the bacterial *attB* site by conventional PCR was performed to determine the absence of inserted Stx phage into *wrbA*, *yehV*, *yecE*, *sbcB*, *Z2577*, *argW*, *prfC*, *ssrA*, and the *torS-torT* intergenic region in each strain. When no *attB* DNA amplification occurred, amplification of the *attL* junction site was performed to demonstrate the presence of inserted Stx phage (Table 1). The amplification reactions were performed in a total volume of 50 μl and contained 0.6 μM primers, 100 μM each deoxynucleoside triphosphate (Roche Diagnostics), 1× PCR buffer with $MgCl_2$, 2.5 U of FastStart-*Taq* polymerase (Roche Diagnostics), and 2 μl of genomic DNA. The reactions were performed in a Veriti thermocycler (Applied Biosystems) with the thermal profiles described in Table 1. The presence of EspK phage inserted in *ssrA* was determined by PCR with primers ssrAF (TGCTGACGAGTGGTTTGTTC) and ssrA-R2 (TGTGATTTCGCTTTTGATGC) for amplification of the 770-bp-long bacterial-EspK phage junction site at the *ssrA* locus. The PCR conditions were as described above, and the thermal profile consisted of an initial denaturation at 94°C for 5 min, followed by 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C for 30 cycles and a final elongation at 72°C for 5 min. PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

To allow rapid and high-throughput analysis of the Stx phage insertion site state at four chromosomal loci, i.e., *wrbA*, *yehV*, *yecE*, and *sbcB*, and to avoid postamplification manipulations, eight quantitative PCR (qPCR) assays were also designed with primers and probes specific for the *attB* and *attL* sites from each locus (Table 1) and compared to the conventional PCR assays. The reactions were performed with the LightCycler 480 instrument (Roche Diagnostics) in a total volume of 20 μl with the thermal profile described in Table 1. The optimal amplification reaction mixture contained 1× LightCycler 480 Probes Master mix (Roche Diagnostics), 200 nM each primer, 200 nM each probe (except for yecE-B, yehV-BL, and yehV-L2, 400 nM), and 2 μl of extracted DNA. The cycle threshold (C_T) value was defined as the PCR cycle at which the fluorescent signal exceeded the background level. The C_T was determined automatically by the LightCycler 480 software by the second derivative maximum method.

For strains containing two different *stx* subtypes, long-template PCR was used to determine at which sites the corresponding two phages were inserted. For this analysis, the LongAmp *Taq* PCR kit (BioLabs) was used with primers stx2-rev (42) and EC11 (37) for a ca. 17-kbp-long stx_2-*yecE* target and primers stx1-rev (42) and yehV-B (43) for a ca. 19-kbp-long stx_1-*yehV* target. The optimal amplification reaction mixture contained 1× LongAmp *Taq* Reaction Buffer (BioLabs), 5 U of LongAmp *Taq* DNA polymerase (BioLabs), 300 μM deoxynucleoside triphosphates (BioLabs), 400 nM each primer, and 1 μl of extracted DNA. The reactions were performed in a Veriti thermocycler (Applied Biosystems) with the thermal profile described in Table 1. PCR products were analyzed by electrophoresis in a 0.8% agarose gel stained with ethidium bromide.

qPCR-based quantification of *attB* and *attL* DNA copies in STEC O26:H11 cultures. qPCR assays targeting the *attB* and *attL* sites at the *wrbA*, *yehV*, *yecE*, and *sbcB* chromosomal loci (Table 1) were used as described above in order to examine the level of spontaneous excision of Stx phages during the cultivation of STEC. The numbers of *attB* and *attL* DNA copies quantified by qPCR for each strain and for each insertion locus were used to calculate *attB*/*attL* ratios (i.e., ratios of bacterial cells whose Stx prophage is excised from the chromosome against those with chromosomally integrated Stx prophage, respectively). The linearity and limit of quantification of each qPCR assay were formerly determined by using calibrated suspensions of STEC corresponding to dilutions of pure cultures of *attL*-positive control strains 11368, H19, 245.2, and VTH7 containing an Stx phage inserted in the *wrbA*, *yehV*, *yecE*, and *sbcB* genes, respectively, and to dilutions of a pure culture of *attB*-positive control strain MG1655 (*E. coli* K-12). Amplification efficiency (E) was calculated with the equation $E = 10^{-1/s} - 1$, where s is the slope of the linear regression curve obtained by plotting the log genomic copy numbers of *E. coli* strains in the PCR against C_T values. The concentrations of DNA samples from the 74 STEC O26:H11 strains of the collection were determined with NanoDrop instruments (Thermo Scientific), and each DNA was then diluted to a fixed concentration of 30 ng/μl prior to qPCR analysis. Student's t test was used to determine whether there were statistically signif-

TABLE 1 Primers and probes for conventional PCR, real-time qPCR, and long-range PCR determinations of Stx phage insertion sites and status of insertion sites as intact (*attB*) or occupied (*attL*)

Target DNA	Primer or probe	Nucleotide sequence (5′→3′)	Amplicon size (bp)	PCR conditions	Reference
Conventional PCR[a]					
wrbA-attB	wrbA1	ATGGCTAAAGTTCTGGTG	600	94°C, 30 s; 59°C, 60 s; 72°C, 60 s	49
	wrbA2	CTCCTGTTGAAGATTAGC			
wrbA-attL	wrbA	CGCCATCCACTTTGCTTG	1,045	94°C, 30 s; 59°C, 60 s; 72°C, 90 s	37
	Int933W	TATGCTACCGAGGCTTGG			
yehV-attB	yehV-A	AAGTGGCGTTGCTTTGTGAT	340	94°C, 30 s; 62°C, 30 s; 72°C, 60 s	43
	yehV-B	AACAGATGTGTGGTGAGTGTCTG			
yehV-attL	yehV-F	CACCGGAAGGACAATTCATC	702	94°C, 30 s; 62°C, 30 s; 72°C, 60 s	43
	yehV-B	AACAGATGTGTGGTGAGTGTCTG			
yecE-attB	EC10	GCCAGCGCCGAGCAGCACAATA	400	94°C, 30 s; 63°C, 60 s; 72°C, 60 s	37
	EC11	GGCAGGCAGTTGCAGCCAGTAT			
yecE-attL	Int258	CATAGCAAACCAAATGGGCCA	425	94°C, 30 s; 57°C, 60 s; 72°C, 60 s	37
	EC11	GGCAGGCAGTTGCAGCCAGTAT			
sbcB-attB	sbcB1	CATGATCTGTTGCCACTCG	1,800	94°C, 30 s; 60°C, 60 s; 72°C, 90 s	49
	sbcB2	AGGTCTGTCCGTTTCCACTC			
sbcB-attL	sbcBF	ATTGTCGCGCTAAAGCTGAT	250	94°C, 30 s; 60°C, 60 s; 72°C, 60 s	25
	stx2cphiB	CAACGATGCTCGTTATGGTG			
Z2577-attB	z2577F	AACCCCATTGATGCTCAGGCTC	909	94°C, 30 s; 59°C, 90 s; 72°C, 60 s	27
	z2576R	TTCCCATTTTACACTTCCTCCG			
argW-attB	argW-A	CCGTAACGACATGAGCAACAAG	216	94°C, 30 s; 58°C, 45s; 72°C, 90 s	32
	argW-D	AATTAGCCCTTAGGAGGGGC			
argW-attL	argW-C	GCATCTCACCGACGATAACA	462	94°C, 30 s; 58°C, 45s; 72°C, 90 s	32
	argW-D	AATTAGCCCTTAGGAGGGGC			
prfC-attB	yiiG1	CCCACCTGGACCGTTTCTTC	348	94°C, 30 s; 55°C, 60 s; 72°C, 60 s	This study
	prfC1	CCCACGCTGCTTTTCCATCT			
prfC-attL	ECO5234	GGAAGAACTGCGGCAGCGAT	914	94°C, 30 s; 56°C, 60 s; 72°C, 90 s	This study
	prfC1	CCCACGCTGCTTTTCCATCT			
torST-attB	torS2	TGCGCGGCGAAAAGTTCCCA	533	94°C, 30 s; 60°C, 60 s; 72°C, 60 s	This study
	torT2	CCGCCTGCCTCCAGCACTTT			
ssrA-attB	ssrA1	GGATTCGACGGGATTTGCGA	838	94°C, 30 s; 55°C, 60 s; 72°C, 90 s	This study
	ypjA-R1	AACGGTATGGAAATTGAGC			
qPCR[b]					
wrbA-attB	wrbA-F1	GCGAATCGCTACGGAATAGA	163	95°C, 10 s (4.4°C/s none)	This study
	wrbA-R1	CGGTACACGCTTAACGACAA		60°C, 30 s (2.2°C/s single)	
	wrbA-B	FAM-CATATTGAAACGATGGCACG-BHQ1		40°C, 30 s (4.4°C/s none)	
wrbA-attL	intW	CCAAAGTGACCAGGAGGATG	200	95°C, 10 s (4.4°C/s none)	This study
	wrbA-R2	GGTGCAGTTTGCGTTTTACC		60°C, 30 s (2.2°C/s single)	
	wrbA-L	FAM-TTAAGCGTGTACCGGAAACC-BHQ1		40°C, 30 s (4.4°C/s none)	
yehV-attB	yehV-F1	AGTGGCGTTGCTTTGTGATA	216	95°C, 10 s (4.4°C/s none)	This study
	yehV-R1	CCGTTCTGCACATCAACATT		60°C, 30 s (2.2°C/s single)	
	yehV-BL	FAM-TTCAACGATGCCGATATTGA-BHQ1		40°C, 30 s (4.4°C/s none)	

(Continued on following page)

TABLE 1 (Continued)

Target DNA	Primer or probe	Nucleotide sequence (5′→3′)	Amplicon size (bp)	PCR conditions	Reference
yehV-attL	yehV-F4	TGTTTACGGAGCATGGATGA	239	95°C, 10 s (4.4°C/s none)	This study
	yehV-R3	TCAATATCGGCATCGTTGAA		60°C, 30 s (2.2°C/s single)	
	yehV-L2	FAM-AAAGTGTCCCATGTATGCCC-BHQ1		40°C, 30 s (4.4°C/s none)	
yecE-attB	yecE-F1	GCAATGGTCGCATCCTAAAT	180	95°C, 10 s (4.4°C/s none)	This study
	yecE-R1	GTCGCCGGAAACTTAAAACA		60°C, 30 s (2.2°C/s single)	
	yecE-B	FAM-GAGTATGCCCGCCACTTTAA-BHQ1		40°C, 30 s (4.4°C/s none)	
yecE-attL	yecE-F4	AGCCAGACTCTGAAATAATATCTTTA	154	95°C, 10 s (4.4°C/s none)	This study
	yecE-R4	AAGCGGAAGTCATCTGTG		60°C, 30 s (2.2°C/s single)	
	yecE-L2	FAM-TAGTTGCCGTCACATTAACTGCGT-BHQ1		40°C, 30 s (4.4°C/s none)	
sbcB-attB	sbcB-F3	ACGGTAAGCAACAATCTA	170	95°C, 10 s (4.4°C/s none)	This study
	sbcB-R4	CTGGGGTAAATAGTCATCC		60°C, 30 s (2.2°C/s single)	
	sbcB-TQB1	FAM-TACGAAACCTTTGGCACGCACC-BHQ1		40°C, 30 s (4.4°C/s none)	
sbcB-attL	sbcB-F4	GGACAATGCTAGACAATGA	192	95°C, 10 s (4.4°C/s none)	This study
	sbcB-R4	CTGGGGTAAATAGTCATCC		60°C, 30 s (2.2°C/s single)	
	sbcB-TQL1	FAM-AGACACAGATAAGCAACCTACCTTCCT-BHQ1		40°C, 30 s (4.4°C/s none)	
Long-template PCR[c]					
stx$_2$-*yecE*	stx2-rev	CTGAACTCCATTAACKCCAGATA	17,000	94°C, 30 s; 60°C, 30 s; 65°C, 19 min	This study
	EC11	GGCAGGCAGTTGCAGCCAGTAT			
stx$_1$-*yehV*	stx1-rev	CGACATYAAATCCAGATAAGAAGTAGT	19,000	94°C, 30 s; 60°C, 30 s; 65°C, 21 min	This study
	yehV-B	AACAGATGTGTGGTGAGTGTCTG			

[a] All PCRs were run for 30 cycles with an initial denaturation step of 5 min at 94°C and a final extension step of 5 min at 72°C.
[b] All qPCRs were run for 40 cycles with an initial denaturation step of 5 min at 95°C (4.4°C/s none). The efficiencies of PCR amplification of *wrbA*, *yehV*, *yecE*, and *sbcB* were 87.1, 96.5, 95.4, and 99.2% for *attL*, respectively, and 96.5, 92.1, 95.4, and 93.6% for *attB*, respectively. None and single indicate the fluorescence acquisition mode selected.
[c] All long-template PCRs were run for 30 cycles with an initial denaturation step of 5 min at 94°C and a final extension step of 10 min at 65°C.

icant differences in the stability of Stx phages according to the insertion site occupied. A *P* value of ≤0.05 was considered a significant difference.

MLST. Multilocus sequence typing (MLST) of 12 *E. coli* O26:H11 strains was performed with the nucleotide sequences of seven housekeeping genes as described previously (37), and the alleles and STs were assigned in accordance with the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The STs of six other strains were retrieved from previous reports (6, 44–46) or from the *E. coli* MLST database.

RESULTS

Identification of *stx* subtypes by PCR. A total of 74 STEC O26:H11 strains were analyzed in this study. They corresponded to 53 *stx*$_1$-positive strains (human, $n = 14$; dairy product, $n = 27$; cattle, $n = 12$), 18 *stx*$_2$-positive strains (human, $n = 15$; dairy product, $n = 3$), and 3 *stx*$_1$- and *stx*$_2$-positive strains (human, $n = 2$; dairy product, $n = 1$). Subtyping of their *stx* genes showed that all of the *stx*$_1$-positive strains harbored the *stx*$_{1a}$ subtype, while all of the *stx*$_2$-positive strains carried the *stx*$_{2a}$ variant, except for one strain (EH196), which carried the *stx*$_{2d}$ gene (Table 2).

Insertion site occupancy by Shiga-toxin bacteriophages in STEC O26:H11 strains. Insertion of Stx phages into nine chromosomal loci, i.e., *wrbA*, *yehV*, *yecE*, *sbcB*, *Z2577*, *argW*, *prfC*, *ssrA*, and *torS-torT*, was determined by conventional PCR tests and by newly developed real-time PCR assays for the first four loci listed above. Identical results were obtained by conventional PCR tests and real-time PCR assays, indicating that the latter can reliably determine Stx phage insertion into the *wrbA*, *yehV*, *yecE*, and *sbcB* genes.

Thirty-seven of the 74 STEC O26:H11 strains studied were found to possess an Stx phage inserted in the *wrbA* gene (Table 2), including 16 human strains, 16 dairy strains, and 5 cattle strains. Twenty-eight strains possessed an Stx phage integrated into the *yehV* gene, including 7 human strains, 15 dairy strains, and 6 cattle strains (Table 2). Ten strains from nine humans and one dairy product and two strains from dairy products possessed an Stx phage integrated into the *yecE* and *sbcB* genes, respectively (Table 2).

In the collection studied here, eight strains (from four humans, three dairy products, and one bovine) possessed two Stx bacteriophages. Of these, three strains carried different subtypes (i.e., *stx*$_{1a}$ and *stx*$_{2a}$) and five strains carried two identical subtypes (i.e., four strains with two copies of *stx*$_{1a}$ and one strain with two copies of *stx*$_{2a}$) (Table 2). By long-template PCR, Stx1a and Stx2a phages were found to be inserted in *yehV* and *wrbA*, respectively, in strain 277.2. In the other two strains, 3073/00 and 3901/97, the Stx2a phage was found to be inserted in *yecE* while the Stx1a phage was located in *yehV* and *wrbA*, respectively. Finally, no Stx phage was found integrated in the *Z2577*, *prfC*, or *argW* gene or in the *torS-torT* intergenic region (data not shown).

Association of *stx* subtypes, Stx phage insertion sites and origins of the STEC strains. Most Stx1a phages were inserted in *wrbA* ($n = 25$) and *yehV* ($n = 27$) genes, with only 4 located in either the *yecE* ($n = 2$) or the *sbcB* ($n = 2$) gene. In contrast, most Stx2a phages were located in the *wrbA* ($n = 12$) and *yecE* ($n = 7$) genes, only one Stx2a phage being inserted in the *yehV* gene. The

TABLE 2 Subtyping of *stx* genes, identification of chromosomal insertion sites for Stx and EspK phages in 74 STEC O26:H11, and determination of *attB*/*attL* ratios

Origin[a] or parameter	Strain	Presence of subtype:			Insertion of Stx phage in[b]:				Insertion of EspK phage in *ssrA*	*attB*/*attL* ratio[c]
		stx_{1a}	stx_{2a}	stx_{2d}	*wrbA*	*yehV*	*yecE*	*sbcB*		
Dairy product	ITFF3406	+			Stx1a				+	1.02×10^{-4}
Dairy product	ITFF3407	+			Stx1a				+	1.06×10^{-4}
Dairy product	ITFF3408	+			Stx1a				+	6.86×10^{-5}
Dairy product	09QMA170.2	+			Stx1a				+	7.65×10^{-5}
Dairy product	09QMA238.2	+			Stx1a				+	3.15×10^{-4}
Dairy product	09QMA277.2[f]	+	+		Stx2a	Stx1a			+	2.34×10^{-3} (*wrbA*); 1.27×10^{-4} (*yehV*)
Dairy product	09QMA283.4	+			Stx1a				+	3.42×10^{-5}
Dairy product	F74-476	+			Stx1a	Stx1a			+	4.28×10^{-5} (*wrbA*); 5.57×10^{-4} (*yehV*)
Dairy product	F46-223[f]		+		Stx2a				+	2.82×10^{-3}
Dairy product	10d[e]	+			Stx1a				+	3.26×10^{-4}
Dairy product	2401-4	+			Stx1a	Stx1a			+	2.29×10^{-5} (*wrbA*); 2.24×10^{-4} (*yehV*)
Dairy product	51.2	+			Stx1a				+	3.44×10^{-4}
Dairy product	F15-313	+			Stx1a				+	2.80×10^{-4}
Dairy product	LA3022401		+		Stx2a				+	2.01×10^{-3}
Dairy product	F43-368		+		Stx2a				+	2.48×10^{-3}
Dairy product	AOC 21.04-4	+			Stx1a				+	2.66×10^{-4}
Dairy product	09QMA245.2	+					Stx1a		+	3.19×10^{-6}
Dairy product	09QMA260.3	+				Stx1a			+	2.86×10^{-7}
Dairy product	2976-1	+				Stx1a			+	1.60×10^{-6}
Dairy product	8102-1	+				Stx1a			+	1.40×10^{-6}
Dairy product	7501 POOLA	+				Stx1a			+	4.85×10^{-4}
Dairy product	158.1	+				Stx1a			+	3.15×10^{-4}
Dairy product	L23A	+				Stx1a			+	2.32×10^{-4}
Dairy product	MAC42.4	+				Stx1a			+	9.07×10^{-8}
Dairy product	175 1A	+				Stx1a			+	1.08×10^{-6}
Dairy product	1028	+				Stx1a			+	2.18×10^{-4}
Dairy product	3591.22	+				Stx1a			+	4.82×10^{-5}
Dairy product	1080.2	+				Stx1a			+	6.33×10^{-4}
Dairy product	979.1	+				Stx1a			+	3.84×10^{-4}
Dairy product	95621-1	+							+	ND[g]
Dairy product	09QMA129.2	+							+	ND
Subtotal no. of strains	31	28	4	0	16	15	1	0	31	
Human (NK)	VTH7	+						Stx1a	+	9.12×10^{-5}
Human (NK)	10003174260	+						Stx1a	+	4.39×10^{-4}
Human (D)	ED21	+			Stx1a				+	1.00×10^{-5}
Human (NK)	96-723	+			Stx1a				+	2.04×10^{-3}
Human (HUS)	31131[f]		+		Stx2a				+	3.65×10^{-3}
Human (HC)	31302	+			Stx1a				+	1.78×10^{-5}
Human (D)	EH284	+			Stx1a				+	3.46×10^{-3}
Human (D)	EH324	+			Stx1a				+	3.34×10^{-3}
Human (D)	H30	+			Stx1a				+	4.35×10^{-6}
Human (HUS)	11368[e]	+			Stx1a				+	4.73×10^{-4}
Human (HUS)	3901/97[e]	+	+		Stx1a		Stx2a		+	9.55×10^{-4} (*wrbA*); 8.78×10^{-5} (*yecE*)
Human (HUS)	5917/97[f]		+		Stx2a				+	2.43×10^{-3}
Human (HUS)	6061/96[f]		+		Stx2a				+	2.92×10^{-3}
Human (HUS)	29348[f]		+		Stx2a				+	2.62×10^{-3}
Human (HUS)	25562		+		Stx2a				+	2.97×10^{-3}
Human (HUS)	30993[e]		+		Stx2a				+	4.99×10^{-3}
Human (HUS)	29246		+		Stx2a				+	2.09×10^{-3}
Human (HUS)	29687		+		Stx2a				+	1.05×10^{-2}
Human (D)	EH196			+			Stx2d		+	1.50×10^{-4}
Human (HUS)	1833/98		+				Stx2a			7.77×10^{-5}

(Continued on following page)

TABLE 2 (Continued)

Origin[a] or parameter	Strain	Presence of subtype:			Insertion of Stx phage in[b]:				Insertion of EspK phage in *ssrA*	*attB*/*attL* ratio[c]
		stx_{1a}	stx_{2a}	stx_{2d}	*wrbA*	*yehV*	*yecE*	*sbcB*		
Human (HUS)	31132[f]		+				Stx2a			1.49×10^{-5}
Human (HUS)	21765(1)[e]		+				Stx2a			3.27×10^{-5}
Human (HUS)	21765(2)		+				Stx2a			7.93×10^{-5}
Human (HC)	31212	+				Stx1a			+	1.07×10^{-5}
Human (HC)	31049	+				Stx1a	Stx1a		+	7.85×10^{-4} (*yehV*); 5.07×10^{-4} (*yecE*)
Human (D)	H19	+				Stx1a				0[d]
Human (HUS)	2245/98[e]	+				Stx1a			+	1.68×10^{-7}
Human (HUS)	3073/00[e]	+	+			Stx1a	Stx2a		+	1.12×10^{-3} (*yehV*); 1.18×10^{-3} (*yecE*)
Human (HUS)	28810	+				Stx1a			+	3.68×10^{-6}
Human (HUS)	7662/96		+			Stx2a	Stx2a			2.52×10^{-3} (*yehV*); 1.01×10^{-4} (*yecE*)
Human (D)	CB6307		+						+	ND
Subtotal no. of strains	31	16	16	1	16	7	9	2	25	
Cattle feces	9	+							+	ND
Cattle feces	130	+								ND
Cattle feces	193	+			Stx1a	Stx1a				1.55×10^{-3} (*wrbA*), 1.16×10^{-3} (*yehV*)
Cattle feces	4	+			Stx1a				+	6.49×10^{-5}
Cattle feces	138	+			Stx1a				+	1.53×10^{-3}
Ground beef	54-126B1	+			Stx1a				+	2.47×10^{-4}
Ground beef	85-08.B	+			Stx1a				+	2.40×10^{-5}
Cattle feces	329S89	+				Stx1a				6.47×10^{-2}
Ground beef	37.40	+				Stx1a			+	3.69×10^{-7}
Ground beef	75136	+				Stx1a			+	3.21×10^{-4}
Cattle feces	19	+				Stx1a			+	4.09×10^{-4}
Cattle feces	113	+				Stx1a			+	3.98×10^{-6}
Subtotal no. of strains	12	12	0	0	5	6	0	0	9	
Total no. of strains	74	56	20	1	37	28	10	2	65	

[a] D, diarrhea; NK, not known.
[b] A total of nine loci were tested for each strain for the presence of an Stx phage by lack of *attB* amplification and positive amplification of *attL*. Only the positive results obtained for four loci are indicated.
[c] The *attB*/*attL* ratio for each Stx phage insertion site was determined by dividing the number of *attB* DNA copies by the number of *attL* DNA copies that were quantified by qPCR.
[d] No *attB* DNA copy was detected.
[e] Strain belongs to ST21.
[f] Strain belongs to ST29.
[g] ND, not done.

sole Stx2d phage identified in STEC O26:H11 was inserted in the *yecE* gene.

By taking the origins of the strains into account, it was observed that dairy and cattle strains possessed mainly an Stx1a phage that was located in either *wrbA* (in 42.8 and 45.5% of the dairy and cattle strains, respectively) or *yehV* (in 53.6 and 54.5% of the dairy and cattle strains, respectively). In contrast, human strains contained either an Stx1a phage or an Stx2a phage in equivalent proportions. In those strains, the Stx1a phage was preferentially integrated in *wrbA* (53.3%) and *yehV* (40%), as opposed to the Stx2a phage, which was preferentially integrated in *wrbA* (50%) and *yecE* (43.7%).

qPCR-based quantification of spontaneous excision of Stx phage DNA in STEC O26:H11 cultures. For some strains, both the *attB* and *attL* sites could be amplified simultaneously, as observed after electrophoresis of PCR products corresponding to several insertion chromosomal loci (data not shown). PCR products originating from *attB* amplification were less abundant, however, than those from *attL* amplification, suggesting that spontaneous excision of Stx prophage DNA occurred in a subset of the STEC cell population.

Such a simultaneous amplification of *attB* and *attL* was also observed by real-time PCR. The C_T values obtained for the *attB* target varied between the strains, suggesting that the amount of cells with excised Stx phage DNA differed according to the strain tested. The C_T values obtained varied from 22.75 to 37.61 for

TABLE 3 Occupancy of various insertion chromosomal loci by Stx phages, EspK phages, or other genetic element in 29 *stx*-negative *E. coli* O26:H11 strains

Origin[a]	Strain	Insertion of Stx phage whose *stx* gene was deleted								Insertion of EspK phage in *ssrA*
		wrbA	*yehV*	*yecE*	*sbcB*	*Z2577*	*prfC*	*torS-torT*	*argW*	
Dairy product	09QMA04.2	−	−	−	−	−	−	−	−	−
Diary product	09QMA315.2	−	−	−	−	−	−	−	−	−
Dairy product	09QMA306.D	−	−	−	−	−	−	−	−	−
Dairy product	FR14.18[d]	−	−	−	−	−	−	−	−	−
Cattle feces	FFL1.1	−	−	−	−	−	−	−	−	−
Cattle feces	FFL2.6	−	−	−	−	−	−	−	−	−
Cattle feces	FV5.36	−	−	−	−	−	−	−	−	−
Cattle feces	FV2.33	−	−	−	−	−	−	−	−	−
Cattle feces	FV3.11	−	−	−	−	−	−	−	−	−
Cattle feces	FV4.17	−	−	−	−	−	−	−	−	−
Dairy product	4198.1	−	−	−	−	−	−	−	−	+
Dairy product	191.1	−	−	−	−	−	−	−	−	+
Dairy product	64.36[c]	−	−	−	−	−	−	−	−	+
Human (HUS)	5021/97	−	+	−	−	−	−	−	−	+
Human (HUS)	5080/97[c]	−	−	+	−	−	−	−	−	+
Human (HUS)	318/98	−	−	−	−	−	−	−	−	−
Human (HUS)	21474	−	−	−	−	−	−	−	−	−
Human (HUS)	21766	−	−	−	−	−	−	−	−	−
Ground beef	19-57D7	−	−	−	−	−	−	−	−	−
Ground beef	V76-1326[d]	−	−	−	−	−	−	−	−	−
Ground beef	39.1	−	−	−	−	−	−	−	−	−
Human (NK)	MB04	−	−	−	−	−	−	−	−	−
Human (NK)	MB01	−	−	−	−	−	−	−	−	−
Dairy product	09QMA355.2	−	−	−	−	−	−	−	−	−
Ground beef	07QMA144.1	−	−	−	−	−	−	−	−[b]	−
Ground beef	07QMA167.1	−	−	−	−	−	−	−	−	−
Ground beef	07QMA184.3	−	−	−	−	−	−	−	−	−
Human (HUS)	29690[d]	−	−	−	−	−	−	−	−	−
Dairy product	F61-523[d]	−	−	−	−	−	−[b]	−	−	−

[a] NK, not known.
[b] Genetic element other than Stx phage inserted.
[c] Strain belongs to ST21.
[d] Strain belongs to ST29.

wrbA-attB, from 26.59 to 41.86 for *yehV-attB*, from 27.25 to 37.92 for *yecE-attB*, and from 28.85 to 34.84 for *sbcB-attB* (data not shown). In comparison, the positive-control MG1655 strain DNA containing intact *attB* sites showed C_T values of 15.7, 16.9, and 16.2 for *wrbA*, *yehV*, and *yecE*, respectively, and strain 11368 with an intact *sbcB-attB* site displayed a C_T value of 15.4 (data not shown).

As this phenomenon can lead to the conversion of STEC to *stx*-negative *E. coli* O26:H11, spontaneous excision of Stx phages was further examined by evaluating the *attB*/*attL* copy number ratio of each strain (Table 2). The amplification efficiencies of the different real-time PCR assays used to quantify *attL* and *attB* genetic copies were similar and were between 87.1 and 99.2% (Table 1). Although the mean *attB*/*attL* ratio was higher for *wrbA* (1.56×10^{-3}) and *yehV* (2.75×10^{-3}) than for *yecE* (2.26×10^{-4}) and *sbcB* (2.65×10^{-4}), these ratios were not statistically significantly different ($P > 0.1$).

Insertion site occupancy by Stx phages or other genetic elements in *stx*-negative *E. coli* O26:H11 strains. Analysis of the *attB* sites by both conventional and real-time PCRs for 29 *stx*-negative *E. coli* O26:H11 strains showed that these were intact, except for four strains (i.e., 5021/97, 5080/97, 07QMA144.1, and F61-523), for which the *attB* site at the *yehV*, *yecE*, *argW*, and *prfC* genes, respectively, was occupied (Table 3). For strains 5021/97 and 5080/97, *yehV-attL* and *yecE-attL* could be amplified by PCR, respectively, suggesting the presence of a phage similar to an Stx phage but whose *stx* gene is deleted. For strains 07QMA144.1 and F61-523, *argW-attL* and *prfC-attL* could not be amplified by PCR, respectively, suggesting that a genetic element other than an Stx phage was present and interrupted the corresponding genes.

Presence of other phages in the *ssrA* site of STEC and *stx*-negative *E. coli* O26:H11 strains. The *attB* site at the *ssrA* gene of most strains could not be amplified, and no Stx phage could be detected at this location, suggesting the presence of another genetic element, such as an EspK phage (33). The presence of such a phage inserted in *ssrA* was therefore investigated here. The *ssrA* gene hosted an EspK phage in 65 out of 74 STEC O26:H11 strains. These included 100, 80.6, and 75% of the dairy product, human, and cattle strains, respectively (Table 2). In contrast, the *ssrA* site was occupied by an EspK phage in a limited number of *stx*-negative *E. coli* O26:H11 strains, i.e., 5 out of 29 (Table 3).

MLST. Phylogenetic analysis of 14 STEC O26:H11 strains was performed by MLST. As described previously for *E. coli* O26:H11 strains (6, 47), the stx_{1a}-positive strains belonged to ST21 whereas strains containing stx_{2a}, either alone or in combination with stx_{1a}, were distributed into both ST21 and ST29 (Table 2). The six *stx*-

negative *E. coli* strains tested by MLST were also found to belong to ST21 and ST29 (Table 3). The correlation between phylogenetic groups and characteristics of STEC O26:H11 such as *stx* genotypes and Stx phage locations was then examined. Seven strains showing six profiles, i.e., stx_{1a}-*wrbA*, stx_{1a}-*yehV*, stx_{2a}-*wrbA*, stx_{2a}-*yecE*, stx_{1a}-*wrbA*/stx_{2a}-*yecE*, and stx_{1a}-*yehV*/stx_{2a}-*yecE*, belonged to ST21, whereas seven other strains that showed the three profiles stx_{2a}-*wrbA* (five strains), stx_{2a}-*yecE*, and stx_{1a}-*yehV*/stx_{2a}-*wrbA* belonged to ST29 (Table 2). The stx_{2a}-*wrbA* and stx_{2a}-*yecE* profiles were therefore each allocated to both STs.

DISCUSSION

Subtyping of the *stx* gene showed that stx_{1a} and stx_{2a} were the major subtypes found in STEC O26:H11 strains, with 56 stx_{1a}-positive and 20 stx_{2a}-positive strains. Three strains contained both stx_{1a} and stx_{2a}, and five strains contained two copies of the same subtype. Similar results were also observed by Bielaszewska et al. in another study of 272 STEC O26 isolates (6). It is noteworthy that most of the dairy strains (88.2%) contained the stx_{1a} gene, whereas the stx_{1a} and stx_{2a} genes were distributed almost equally in the human strains. No other *stx* subtype was found here, except for the stx_{2d} subtype in one human strain, which has been reported recently in emerging STEC O26:H11 human strains (45).

A total of four genes (i.e., *wrbA*, *yehV*, *yecE*, and *sbcB*) were used as Stx phage chromosomal insertion loci in most of the STEC O26:H11 strains, with *wrbA* and *yehV* being the major insertion sites. In the five remaining STEC strains, none of the nine insertion sites tested here were occupied by an Stx phage, whose location therefore remains to be determined. Other candidates for insertion sites, which were not tested here, could be the *potC*, *yciD*, *ynfH*, *serU*, and *yjbM* genes (48).

All of the STEC O26:H11 strains from dairy products and cattle possessed Stx phages integrated into *wrbA* or *yehV*, except for one strain that contained an Stx1a phage located in *yecE*. The *wrbA* and *yehV* genes also served as Stx phage insertion sites in the human strains. Compared to dairy and cattle strains, more human strains (i.e., $n = 9$, 28%) carried an Stx phage located in *yecE*. To our knowledge, only the integration of Stx2 phages into *wrbA* and *yecE* was already described elsewhere for STEC O26:H11 (37). In our study, all of the strains that possessed an Stx2a phage integrated in *wrbA* and *yecE* caused HUS, which is indicative of high virulence. Interestingly, such a profile was either absent from or rarely identified in the dairy or cattle strains studied here. In addition, *yecE*-located Stx2a and Stx2d phages and *sbcB*-located Stx1a phage were found only in human strains. However, as the number of strains tested here is limited, it is premature to conclude about the absence of STEC O26:H11 harboring such Stx phages in dairy products.

MLST-based phylogenetic analysis of 20 *E. coli* O26:H11 strains showed that the 14 STEC isolates tested belonged to either ST21 or ST29, as described previously (6). The stx_{2a}-positive *E. coli* O26:H11 strains tested that caused HUS were distributed in the ST21 and ST29 subgroups, in agreement with previous findings showing that stx_{2a} rather than the ST is a predictor of HUS development (6). In addition, combinations of an *stx* genotype with an insertion locus (e.g., stx_{2a}-*wrbA* or stx_{2a}-*yecE*) could be assigned to both phylogenetic subgroups, therefore indicating that they did not necessarily correlate with a particular ST. This is not surprising, however, since Stx phages are mobile genetic elements acquired horizontally. The remaining six *stx*-negative *E. coli* O26:H11 strains typed by MLST also belonged to ST21 or ST29, as previously observed (47), suggesting interconversion between STEC and *stx*-negative *E. coli* O26:H11 by loss or gain of Stx phage.

In investigating the origin of *stx*-negative *E. coli* O26:H11, we found that both *attL* and *attB* could be detected simultaneously in STEC O26:H11 genomic DNA extracts, as has been observed previously for STEC O157:H7 (43). This suggests that spontaneous excision of Stx phage DNA occurred in a subset of STEC cells during growth. However, such instability of Stx prophage DNA was not dependent on the insertion site since no significant difference could be identified between the mean *attB*/*attL* ratios calculated for each chromosomal insertion site. Whether spontaneous excision of Stx prophage DNA contributes to loss of Stx phage and concomitant conversion *in vitro* and *in vivo* to *stx*-negative *E. coli* O26:H11 strains (37, 38) remains to be further elucidated.

In addition, apart from *ssrA*, all of the chromosomal bacterial attachment sites were found to be vacant in all *stx*-negative *E. coli* O26:H11 strains, except for four strains, indicating that absence of the *stx* gene from most *stx*-negative *E. coli* O26:H11 strains was due to the absence of Stx phage and not to a deletion within the Stx prophage, as observed for an O103:H25 strain (38, 46). In the remaining four *stx*-negative *E. coli* O26:H11 strains, one of the *attB* sites was found to be interrupted, most probably by an Stx phage whose *stx* gene was deleted or by another genetic element.

Finally, a prophage encoding the type III effector EspK was located in the *ssrA* gene in the majority (87.8%) of STEC O26:H11 strains. In contrast, this EspK prophage was observed in only 17.2% of the *stx*-negative *E. coli* O26:H11 strains studied. These observations are in agreement with those of Bugarel et al. showing the presence of the *espK* gene in EHEC O26 strains and their derivatives but not in *stx*-negative *E. coli* O26:H11 strains (33). Most of the *stx*-negative *E. coli* O26:H11 strains studied here thus differed from STEC O26:H11 by the absence of two genetic elements, i.e., an Stx prophage and an EspK prophage. Whether these *stx*-negative *E. coli* O26:H11 strains stem directly from STEC O26:H11 by spontaneous loss of these two phages is unknown. Alternatively, these *stx*-negative *E. coli* O26:H11 strains might not be STEC O26:H11 derivatives.

Conclusion. In conclusion, a diverse range of genetic patterns was observed among STEC O26:H11 strains isolated from dairy products, cattle, and human patients. Various *stx* subtypes and insertion sites were identified among the Stx phages that lysogenized STEC O26:H11, with some differences observed between human strains and strains from food and cattle. These results confirm previous reports showing the existence of different clones (or clades) of STEC O26:H11 with various levels of pathogenicity.

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